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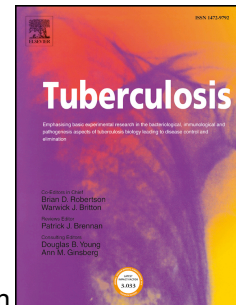
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**Title page:** short communication

The impact of repeated NALC/NaOH- decontamination on the performance of Xpert MTB/RIF assay

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Running title: NALC/NaOH-decontamination and Xpert MTB/RIF-assay

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## Summary

The Xpert MTB/RIF assay detects *Mycobacterium tuberculosis* in unprocessed or NALC/NaOH- decontaminated sputum. The effect of repeated NALC/NaOH- decontamination on several Xpert performance parameters was assessed in this study. A second NALC/NaOH-decontamination had no effect on the binary Xpert-outcome but increased the value for the quantitative readout ( $C_{Tmin}$ ). Repeated decontamination was not associated with PCR-inhibition or invalid results. The  $C_{Tmin}$  of M.tb positive samples was higher in inhibited Xpert runs. Our data indicate that NALC/NaOH-decontamination has an effect on the performance of the Xpert assay, and that  $C_{Tmin}$  readouts of decontaminated sputum samples should be interpreted with caution.

44 **Keywords:** Xpert-inhibition; sputum processing; quantitative PCR; Xpert-performance

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## Introduction

The Xpert MTB/RIF assay (Xpert) is a fully automated PCR test [1], which has been endorsed by WHO for the rapid detection of *Mycobacterium tuberculosis* (*M.tb*) in sputum samples. As previously published, fresh unprocessed sputum as well as NALC/NaOH-decontaminated, concentrated sputum pellets can be used for Xpert testing without effecting the assay's sensitivity to detect *M.tb* [2]. While Xpert testing is usually performed on unprocessed sputum samples in most clinical settings, decontaminated sputum samples are often used in the context of research and Xpert evaluation studies [3-5]. It is not unusual for sputum samples to require repeated decontamination due to bacterial overgrowth in culture. The impact of NALC/NaOH-decontamination on Xpert's capacity to detect *M.tb* are scarce apart from the study by Boehme et al. [2]. Furthermore, only a few studies have analysed the inhibition of Xpert in sputum and extrapulmonary samples [6-9]. Blakemore and colleagues showed that inhibition of the Xpert assay resulted in an increased  $C_{Tmin}$  for *M.tb*- positive samples and poorer correlation with the quantitative readouts of the standard methods [10]. Although the typical inhibitors are usually specific to sample type and related characteristics, insoluble calcium-phosphate crystals, formed during the NALC/NaOH-processing, were also reported to inhibit DNA amplification [11]. In this study we analysed the effect of repeated decontamination on critical performance parameters of Xpert such as detection of *M.tb* and PCR-inhibition.

## Results and Discussion

We used serial sputum samples from initially smear positive patients enrolled into a phase III TB treatment trial (ClinicalTrials.gov: NCT00864383) in Mbeya, Tanzania. Comprehensive information on ethical aspects, study design and laboratory methods have been published elsewhere [3, 12]. Briefly, after the samples were homogenized and decontaminated, the resulting pellet was processed for smear microscopy and culture methods and the residual was stored at 4°C. If the first culture result was lost due to contamination within the first ten days after inoculation the sputum samples were decontaminated for a second time.

A total of 1134 once or twice decontaminated sputum samples from 90 patients were stored at -80°C until Xpert testing (GeneXpert Dx version 2.1 and 4.0). 97.62% of all

77 samples (1107/1134) had a final Xpert result (Figure 1), and 38.8% (440/1134) of  
78 samples were decontaminated twice -out of which seven had an invalid result- with a  
79 non-significant trend ( $p= 0.063$ ) towards a higher proportion of repeated decontamination  
80 for samples from the later study time points.

81 The Xpert test includes a sample processing control (SPC), to confirm adequate  
82 processing and to detect the presence of inhibitors. A negative result for SPC indicates an  
83 invalid test in *M.tb*-negative samples. In *M.tb*-positive samples a PCR-run with a CT  
84 value of  $>34$  for the SPC is considered as inhibited [10]. As shown before by Blakemore  
85 at al. [10], we confirmed in this study that in Xpert-positive samples with a  $C_T > 34$  for  
86 SPC the readout for minimal cycle threshold ( $C_{Tmin}$ ) was significantly increased ( $p$   
87  $< 0.001$ ) compared to samples with a  $CT < 34$  for the SPC (data not shown). We further  
88 questioned whether Xpert-inhibition could be caused by NALC/NaOH-decontamination  
89 and found no data to support this idea. In fact, the risk for an invalid or inhibited Xpert  
90 runs was even reduced by 32% and 18%, respectively, after repeated decontamination.  
91 Although this observation was not statistically significant in our study (Table 1), it is in  
92 line with other reports, indicating that additional samples processing steps such as sample  
93 washing, centrifugation or filtration may reduce inhibition of Xpert [6-8].

94 The GeneXpert software reports qualitative test results as well as semi quantitative  
95 readouts based on the  $C_{Tmin}$  determined by one out of five probes specific for *M.*  
96 *tuberculosis* complex becoming positive first. In this study we were investigating the  
97 effect of NALC/NaOH-decontamination on the qualitative and quantitative Xpert  
98 MTB/RIF result. The analysis of the 1107 samples with a final Xpert result showed that  
99 there was no difference in the binary outcome, TB positive or negative, between samples  
100 that had been decontaminated once or twice (Table 1). This is consistent with recent  
101 findings, indicating that the assay's sensitivity for *M. tb* was not affected regardless  
102 whether fresh or NALC/NaOH-decontaminated sputa were used [2]. We expand this  
103 observation to find out whether repeated decontamination had any effect on the bacterial  
104 load measured by the  $C_{Tmin}$ . Our analysis of 880 samples with a *M.tb*-positive Xpert result  
105 revealed that duplicate decontamination did have a significant effect on the reported  
106 bacterial load of a sample, expressed as the number of cycle thresholds ( $C_{Tmin}$ ) and the  
107 semi-quantitative readouts (Table 1). The overall median for  $C_{Tmin}$  in twice versus once

decontaminated samples was significantly higher, 23.8 versus 21.5 ( $p < 0.001$ ), and this might be interpreted as a lower bacterial load. Further, after adjusting for week after treatment initiation in the regression model, the risk for the detection of “high” bacterial burden measured by Xpert was reduced by 42% (RR= 0.58, 95%CI: 0.40 to 0.86,  $p = 0.007$ ) for twice contaminated samples, whereas the risk was increased by 50% (RR= 1.50, 95%CI: 1.10 to 2.04,  $p = 0.010$ ) for detecting a “very low” bacterial burden. The lower risk for detection of “high” amount of DNA in connection with a greater risk for detection of “very low” amount of bacterial DNA for twice decontaminated samples suggest, that the amount of detectable DNA is reduced by a second decontamination step. Loss of apparent bacterial load following NALC/NaOH- decontamination is well recognized for a culture end point [13-14]. Unlike other PCR-assays [14], the Xpert assay was designed to detect only DNA that is associated with viable mycobacteria, which is achieved by an integrated mesh and washing step [1]. Yet, we have shown that DNA linked to non-viable bacilli is also detected by Xpert [3]. The data of this study suggest, that the second decontamination appears to render some mycobacterial DNA undetectable by Xpert in these samples. Although we were not comparing fresh sputum results with data of single decontaminated samples, it is likely that a similar effect of NALC/NaOH decontamination will be detectable in such comparison. This, however, needs confirmation by further studies.

In addition to the previous publication by Devonshire et al. [15], which showed that there is a huge variability among Xpert  $C_{Tmin}$  data generated from different GeneXpert instruments, these data provide additional evidence that Xpert  $C_{Tmin}$  or semi-quantitative readouts should be used with great caution, when they are used for allocation into different arms or comparison of treatments within clinical trials or for judgments regarding disease progression, infectiousness or response to anti-TB treatment within clinical settings. Uncritical application of NALC/NaOH-decontamination in the laboratory or unawareness about these procedures among clinicians and scientist might lead to misinterpretation of quantitative Xpert data and underestimation of mycobacterial load. These data further suggest that twice decontaminated sputum samples should be excluded from the interpretation of quantitative Xpert results [3-5]. Future evaluation studies of Xpert or up-coming newer versions of this test should include an assessment of

139 the effect of different sputum processing steps on the relevant diagnostic performance  
140 parameters of this assay.

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**TABLE 1:** The effect of repeated NALC/NaOH-decontamination on Xpert results

	Re- Decontamination		RR*	95%CI for RR*	p-value*
	Yes	No			
Xpert positive % , n=1107 <sup>†</sup>	76.7 (332/433)	81.3 (548/674)	0.98	0.92 to 1.05	0.594
Xpert semi-quantitative result, n=880 <sup>‡</sup>					
high %	7.8 (26/332)	14.8 (81/548)	0.58	0.40 to 0.86	0.007
medium %	30.7 (102/332)	40.3 (221/548)	0.80	0.65 to 0.99	0.037
low %	37.7(125/332)	30.7(168/548)	1.22	0.96 to 1.54	0.099
very low %	23.8 (79/332)	14.2 (78/548)	1.50	1.10 to 2.04	0.010
Xpert CTmin (overall median), n=880 <sup>‡</sup>	23.8	21.5	1.07	1.04 to 1.11	<0.001
Invalid PCR run (SPC0= 0) %, n=1134 <sup>§</sup>	4.1 (18/440)	6.1 (42/694)	0.68	0.36 to 1.29	0.237
PCR inhibited (SPC > 34), %, n=1071 <sup>  </sup>	8.5 (36/422)	10.6 (69/649)	0.82	0.57 to 1.18	0.283

RR: risk ratio, 95%CI: 95% confidence interval, Xpert: Xpert MTB/RIF assay, SPC: sample processing control, PCR: polymerase chain reaction

\* Adjusted for week after treatment initiation

<sup>†</sup> 1107 samples with a valid Xpert result were included in the analysis

<sup>‡</sup> 880 samples with an *M. tuberculosis* positive Xpert result were included in the analysis

<sup>§</sup> Total amount of 1134 samples from 90 smear positive TB patients were included in this analysis

<sup>||</sup> 1071 samples with a positive result for SPC (SPC ≠ 0) were included in this analysis

203 **FIGURE 1**

204 Title: Sample flow chart

205 Legend: The sample chart is reflecting all 1134 samples which were included in the study  
206 and their distribution to different subgroups for analysis.

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Title: Sample flow chart

Legend: The sample chart is reflecting all 1134 samples which were included in the study and their distribution to different subgroups for analysis.

